Glucocorticoid adrenal steroids and glucocorticoid-inducible kinase isoforms in the regulation of GluR6 expression

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Generation of memory is enhanced during stress, an effect attributed to stimulation of neuronal learning by adrenal glucocorticoids. The glucocorticoid-dependent genes include the serum- and glucocorticoid-inducible kinase SGK1. SGK1 is activated through the phosphatidylinositol 3 kinase (PI3-kinase) pathway by growth factors such as insulin-like growth factor-1 (IGF1) or tumour growth factor β (TGF-β). Previously, a fourfold higher expression of SGK1 has been observed in fast-learning rats as compared with slow-learning rats. The mechanisms linking glucocorticoids or SGK1 with neuronal function have, however, remained elusive. We show here that treatment of mice with the glucocorticoid dexamethasone (238 μg day^{-1} for 8–20 days) enhances hippocampal expression of GluR6. Immunohistochemistry reveals significantly enhanced GluR6 protein abundance at neurones but not at astrocytes in mice. Immunohistochemistry and patch clamp on hippocampal neurones in primary culture reveal upregulation of GluR6 protein abundance and kainate-induced currents following treatment with dexamethasone (1 μM) and TGF-β (1 μM). In *Xenopus* oocytes expressing rat GluR6, coexpression of SGK1 strongly increases glutamate-induced current at least partially by increasing the abundance of GluR6 protein in the plasma membrane. The related kinases SGK2 and SGK3 similarly stimulate GluR6, but are less effective than SGK1. The observations point to a novel mechanism regulating GluR6 which contributes to the regulation of neuronal function by glucocorticoids.

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Kainate receptors (KARs) belong to the ionotropic glutamate receptors (GluRs). They contribute to the excitatory postsynaptic potential in response to glutamate, and modulate the release of neurotransmitters (GABA and glutamate) through presynaptic mechanisms (Chittajallu *et al.* 1999; Mulle *et al.* 2000; Contractor *et al.* 2001). KARs are regulated during brain development and by neuronal activity (Kidd & Isaac, 1999). The mammalian KAR gene family consists of five subunits: GluR5, 6 and 7 (low-affinity KARs), and KA1 and KA2 (high-affinity KARs). The KAR subunit GluR6 is abundantly expressed in brain regions involved in learning and memory, such as the hippocampus, as well as in cerebral structures serving motoric and motivational aspects of behaviour, such as basal ganglia and cerebellum (Mulle *et al.* 1998). Recently, it has been shown that postsynaptic KARs contribute to the excitatory postsynaptic current (EPSC) at different types of synapses (Frerking & Nicoll, 2000; Lerma *et al.* 2001). The KAR-mediated EPSCs have small amplitudes and show mostly slow kinetics. Genetic deletion of GluR6 (Mulle *et al.* 1998) has revealed important and distinct roles for the GluR6 subunit in synaptic transmission and plasticity in the hippocampus (Bureau *et al.* 1999; Contractor *et al.* 2000; Contractor *et al.* 2001; Huettner, 2001). Little is known about regulation of GluR6. Earlier studies pointed to modulation of GluR6 activity by protein phosphorylation (Wang *et al.* 1993). However, the protein kinase involved has not been defined.

Among the potential candidate kinases is the serum- and glucocorticoid-inducible kinase SGK1 (Webster *et al.*

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1993*a*,*b*). This kinase has been implicated in the generation of memory, since, in rats, SGK1 expression correlates with the ability to learn (Tsai *et al.* 2002). The kinase is expressed in all human tissues tested thus far (Waldegger *et al.* 1997), including the brain (Wärntges *et al.* 2001). Expression of the kinase is upregulated by glucocorticoids (Firestone *et al.* 2003), which are known to facilitate memory consolidation and memory storage in a variety of learning tasks in mice and rats (Tsai *et al.* 2002). Tsai and coworkers further showed that SGK1 RNA is expressed in the rat hippocampus (Tsai *et al.* 2002). SGK1 is highly expressed in the CA1 cell layer, CA3 cell layer and gyrus dentatus (Tsai *et al.* 2002), hippocampal regions that similarly express high levels of GluR6 (Bureau *et al.* 1999; Frerking & Nicoll, 2000).

Among the well-established functions of SGK1 is the stimulation of the renal epithelial Na^+ channel ENaC by increasing the abundance of the channel protein in the plasma membrane (Lang *et al.* 2003; Pearce, 2003; Verrey *et al.* 2003). This function is shared by the related kinases SGK2 and SGK3 (Friedrich *et al.* 2002), which are similarly expressed in the brain (Kobayashi & Cohen, 1999). Unlike SGK1, SGK2 and SGK3 are not under transcriptional control of glucocorticoids (Lang & Cohen, 2001). All three kinases are activated by a signalling cascade triggered by phosphatidylinositol 3 kinase (PI3-kinase), and are regulated by insulin and growth factors such as insulin-like growth factor-1 (IGF1), brain-derived neurotrophic factor (BDNF) and tumour growth factor β (TGF-β) (Lang & Cohen, 2001). The present study has been performed to elucidate the regulation of the KAR subunit GluR6 by glucocorticoid hormones, and the kinases SGK1, SGK2 and SGK3.

Methods

cRNA synthesis

Template DNA was linearized with a suitable restriction enzyme. cRNA was synthesized from 1 μg of linearized DNA using an *in vitro* transcription kit (mMessage mMachine T7 kit; Ambion). cRNA concentrations were evaluated by photospectrometry and transcript quality was checked by agarose gel electrophoresis.

RT-PCR analysis

Total RNA was isolated from tissue by using the Qiashredder and RNeasy Mini Kit from Qiagen. For cDNA first-strand synthesis, 1 μg of total RNA in 12.5 μl diethylpyrocarbonate (DEPC)-H_2O was mixed with 1 μl of oligo-dT primer (500 μg ml^{-1}; Invitrogen, Karlsruhe, Germany) and heated for 2 min at 70°C. A RT mix of 2 μl 10× reaction buffer (Biolabs, MA, USA), 1 μl dNTP mix (dATP, dCTP, dGTP, dTTP, 10 mM each, Promega), 0.5 μl recombinant RNase inhibitor (Roche), 0.1 μl M-MuLV reverse transcriptase (Biolabs) and 2.9 μl DEPC-H_2O was then added, and the reaction mixture was incubated for 60 min at 42°C. The reaction was stopped by heating the mixture for 5 min at 94°C. The cDNA was stored at −20°C prior to PCR analysis. PCR analysis was then performed with 1 μl of the reverse transcription product in a total volume of 25 μl of a PCR mix containing 22 μl of sterile H_2O, 1 μl of primer 1 (10 pmol μl^{-1}), 1 μl of primer 2 (10 pmol μl^{-1}) and one puReTaq Ready-To-Go PCR bead (Amersham Biosciences, Freiburg, Germany) through 40 cycles (30 s at 94°C, 30 s at 60°C, 45 s at 72°C). The following primers were used to amplify a 271 bp stretch of the Sgk1 isoform: sense primer: 5′-TGAAACAGAGAAGGATGGGC-3′; antisense primer: 5′-TTGTGCCTAGCCAGAAGAAC-3′. PCR products were analysed by agarose gel electrophoresis.

Electrophysiological measurements in *Xenopus* oocytes

Oocytes of stages V–VI were surgically removed from the ovaries of *Xenopus laevis*, as described elsewhere (Seebohm *et al.* 2003). Female *Xenopus laevis* frogs were anaesthetized with 0.1% tricaine (Sigma-Aldrich, Deisenhofen, Germany), and pieces of ovary were surgically removed. The incisions were sutured and the animals allowed to recover. Frogs were humanely killed after the final collection (Maljevic *et al.* 2003). The experimental procedures were approved by the Regierungspraesidium Tuebingen, Germany. Oocytes were injected with 4 ng of GluR6 cRNA with or without 6 ng SGK/protein kinase B (PKB) cRNA using a Nanoliter 2000 injector (WPI, Inc., FLA, USA). Standard two-electrode voltage-clamp recordings were performed 5–8 days after cRNA injection with a TurboTec 03 amplifier (npi, Tamm, Germany) and an interface DIGIDATA 1322 A from Axon Instruments. Data analyses were done with pClamp 9.0/clampfit 9.0 software (Axon Instruments) and Origin 6.0 software (Microcal). Agonist solutions were prepared in ND-96 buffer (mM: NaCl 96, $CaCl_2$ 1.8, KCl 2.0, $MgCl_2$ 1.0 and Hepes–NaOH 5, pH 7.2 with NaOH). Current and voltage electrodes were filled with 3 M KCl and had resistances of 0.5–1.5 MΩ. The oocytes were held at −70 mV and agonist (300 μM glutamate; Sigma-Aldrich) was applied by superfusion for ∼10 s at a flow rate of 10–14 ml min^{-1}. Prior to agonist application, the oocytes were incubated for 8 min in concanavalin A (ConA) to prevent desensitization.

Labelling of cell surface proteins using biotinylated ConA

To identify the fraction of receptor protein inserted in the plasma membrane, surface proteins were tagged with biotinylated ConA (Sigma-Aldrich), and isolated

by streptavidin/sepharose-mediated precipitation of the biotinyl-ConA/protein complex, as described elsewere (Strutz *et al.* 2002). Briefly, intact oocytes were incubated in 10 μM biotinyl-ConA (Sigma-Aldrich) for 30 min at room temperature. At this step, ConA binds to glycosylated plasma membrane proteins, e.g. glutamate receptors. Since intact oocytes were used, only plasma membrane proteins are labelled and not internal membrane proteins. After five 10 min washes in ND-96 buffer to remove excess ConA, 20 intact oocytes were homogenized with a Teflon pestle in H-buffer (20 μl oocyte^{-1}; 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, plus a mixture of proteinase inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany)) and were kept at 4°C for 1 h on a rotating rod. A 20 μl aliquot was kept as a total protein sample (T). After centrifugation of the remaining homogenate for 1 min at 16 000 *g*, the supernatants were supplemented with 20 μl of washed streptavidin/sepharose beads (Sigma-Aldrich) and incubated at 4°C for 3 h on a rotating rod. During this step the streptavidin beads bound to the biotinylated ConA–plasma membrane receptor complex. The streptavidin/sepharose beads were then pelleted by a 2 min spin at 16 000 *g*, and washed three times in H-buffer. A 20 μl aliquot of the supernatant was kept as supernatant protein sample (SN). The final pellets (P) containing plasma membrane receptors were boiled in 20 μl of SDS-PAGE loading buffer (0.8 M β–mercaptoethanol, 6% SDS, 20% glycerol, 25 mM Tris-HCl, pH 6.8, and 0.1% bromophenol blue).

Animals

Age and sex-matched siblings of Sv129J mice (2–3 months old) were anaesthetized intraperitoneally with ketamine (100 mg (kg body weight)$^{-1}$; Sigma-Aldrich) and xylazine (4 mg (kg body weight)$^{-1}$; Sigma-Aldrich) prior to the subcutaneous implantation of a placebo or dexamethasone (DEX) pellet (both from Innovative Research of America, Sarasota, USA). DEX pellets (5 mg pellet^{-1}) had a continuous and linear release of 238 μg DEX per day, and were used for either 8 or 20 days. To obtain brain tissue, mice, anaesthetized with the aforementioned mixture, were killed after loss of pedal reflexes, and terminally bled into the thoracic cavity and placed on ice. The brain was then taken out of the skull and immediately frozen in liquid nitrogen (Strutz-Seebohm *et al.* 2005). The experimental protocols were approved by the local governmental council for animal care and were conducted according to the German law for the care and use of laboratory animals.

Gel electrophoresis and Western blotting

Proteins from homogenized oocytes were separated by SDS electrophoresis and transferred to nitrocellulose filters. Blots were blocked in 1× PBS containing 5% milk powder for at least 1 h at room temperature. For the detection of GluR6, primary rabbit immunoaffinity purified anti-GluR6 antibody (1 μg μl^{-1}; Upstate/Biomol, Hamburg, Germany) and secondary horseradish-peroxidase-conjugated donkey anti-rabbit antibody (1:1000 dilution; Amersham Biosciences) were used. For verification of protein levels, Ponceau Red staining was performed.

Immunohistochemistry

Anaesthetized animals were fixed by cardiac perfusion with 4% paraformaldehyde. Brains were quickly removed and postfixed for 2 h in the same fixative. After rinses in PBS and cryoprotection in 30% sucrose, brain sections were taken using a cryostat at 20 μm and mounted on gelatinized slides. Hippocampal primary cultures were fixed for 30 min in 4% paraformaldehyde, and then stained as for the sections. Sections of the hippocampal region were washed twice in PBS for 15 min at room temperature, followed by preincubation in a solution of 4% normal goat serum, 1% bovine serum albumin and 0.25% Triton X-100, in PBS (all from Sigma-Aldrich) for 1 h. All subsequent washes and dilutions of staining compounds were performed in PBS containing 0.3% Triton X-100 and 1% DMSO. After preincubation, primary rabbit anti-GluR6 antiserum (diluted 1:200; Upstate/Biomol, catalogue no. 06309) was applied to the sections in a moist chamber overnight at 4°C. Following three washes of 10 min each, the sections were covered with goat anti-rabbit Alexa-Fluor-488-conjugated secondary antibody (1:400; Molecular Probes/Invitrogen, Karlsruhe, Germany) for 1.5 h at room temperature. As a counterstain, sections were finally incubated in fluorescent Nissl stain (Neuro Trace red, Molecular Probes/Invitrogen) in 1% albumin in PBS for 2 h at room temperature. After three final washes in PBS they were coverslipped in Fluorosave (Calbiochem/Merck, Darmstadt, Germany). In some cases, cultures and cryostat sections were also stained with primary antibodies against either glial fibrillary acidic protein (GFAP) or microtubule-associated protein-2 (MAP-2; Sigma-Aldrich; both mouse monoclonal) specific for astrocytes and neurones, respectively. The monoclonal antibodies were detected by anti-mouse Alexa Fluor-660 secondary antibodies. Analysis of the sections was performed on a confocal microscope (Zeiss LSM 510, Jena, Germany) using the argon laser excitation wavelength at 488 nm and a He–Ne laser at 543 nm (for fluorescent Nissl stain), or a He–Ne laser at 633 nm for double immunostains, with appropriate filter sets for detection and the system's multitrack function. When immunostains were compared quantitatively, high-power images (×40 oil objective, NA = 1.3, zoom 4.1) with

only 488 nm excitation were scanned from sections from treated and untreated animals stained in parallel. Laser and detector settings were identical, and confocal image stacks were generated covering a range of 3.5 μm in the z direction. Thus, to determine differences in staining, pixel intensities in a tissue volume of $10 \times 10 \times 3.5$ μm from image stacks were analysed using the LSM510 software. Intensities were compared after subtraction of background staining from nonsynaptic regions such as myelinated fibre tracks. Stains were performed at least three times for each sibling pair of DEX-treated and untreated (sham) mice.

Primary culture of hippocampal neurones

Embryonic mice of 15 days gestational age (E15) were prepared from timed-pregnant C57/BL6 mice anaesthetized with ketamine/xylazine. After removal of embryos, anaesthetized mothers were killed by cervical dislocation. Embryos were kept in cold HBSS (4°C), removed from the amnionic sac and craniotomized. Hippocampi were microdissected and incubated in Hanks' balanced salt solution (Sigma-Aldrich) containing 0.05% DNase and 0.05% trypsin for 20 min. After blocking with fetal calf serum (Gibco/Invitrogen, Karlsruhe, Germany), tissues were dissociated by trituration using a fire-polished pipette to get a single cell suspension. A quantity of 100 000 cells cm^{-2} were seeded onto poly-L-ornithine (100 $\mu g\,ml^{-1}$, Sigma-Aldrich)–fibronectin (50 $\mu g\,ml^{-1}$, Gibco/Invitrogen)-coated culture dishes in culture medium, and kept in a humidified incubator at 37°C in 95% O_2/5% CO_2. The culture medium consisted of DMEM/F12 (2:1), B27 (1:50), 1% fetal calf serum, and 1% penicillin/streptomycin (all from Gibco/Invitrogen), and was changed every 2–3 days. Culture of neural cells was carried out up to 2 weeks. Twenty hours prior to electrophysiological experiments, serum was removed and DEX (1 μM; Sigma-Aldrich) with or without TGF-β (1 μM; Sigma-Aldrich) added to the culture medium.

Electrophysiological recordings from hippocampal neurones

Whole-cell patch-clamp recordings obtained at a clamp voltage of −70 mV were 3 kHz low-pass filtered using an EPC-9 amplifier (HEKA, Lambrecht, Germany). Borosilicate glass pipettes (GC 150 TF-10; Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS 314 (Märzhäuser, Wetzlar, Germany) electrical micromanipulator. Data acquisition and analysis were performed by Pulse software. Whole-cell patch-clamp recordings were made at room temperature using 3–7 MΩ patch pipettes with an internal solution containing (mM) 135 CsCl, 3 $MgCl_2$, 2.5 EGTA, 10 Hepes–CsOH, and 1 Na_2ATP, pH 7.4. The cells were continuously superfused with external solution containing (mM) NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, Hepes–NaOH 10, and glucose 5, pH 7.4. Agonist kainate (KA; 100 μM) and a combination of KA (100 μM) and 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX) (1 μM) or SYM 2206 (100 μM) were applied to the bath. Cells were incubated in ConA (100 μM) for 10–15 min before being transferred to the recording chamber. KA and NBQX were obtained from Tocris/Biotrend, Köln, Germany; SYM2206 and ConA were obtained from Sigma-Aldrich. Current amplitudes were normalized to the cell capacitance (i.e. pA pF^{-1}) to account for different cell surface areas.

Statistical analysis

For the immunoblotting studies, representative immunoblots are shown, and a quantitative assessment of plasma membrane abundance was carried out by densitometric analysis (Scion Image software) of immunoblots from similar experiments. Before pooling the results from different blots, the result from each blot was expressed as a percentage of the control value (relative abundance). The combined results from all blots were then expressed as means ± S.E.M. Statistical analysis of the data was performed by Origin 6.0. Student's t test was applied for unpaired data and $P < 0.05$ was considered statistically significant. Oocyte experiments were analysed by Student's t test or ANOVA, as applicable. Electrophysiological data are presented as means ± S.E.M. (n = number of cells). Statistical significance of differences between means was defined by ANOVA.

Results

Brain sections stained with anti-GluR6 polyclonal antibody showed strong staining in the dentate gyrus and CA3 synaptic layers of the mouse hippocampus (Fig. 1*A*). Double stains for GluR6 and GFAP specific for astrocytes, and for GluR6 and neurone-specific MAP-2, showed that GluR6 is expressed by both astrocytes and neurones (data not shown). Administration of the glucocorticoid DEX for 8 or 20 days led to a significant increase of GluR6 immunofluorescence in the mouse hippocampus (Fig. 1*B* and *C*). In astrocytes, GluR6 abundance was not enhanced in DEX-treated animals as compared with control (untreated/sham) animals. This result is consistent with predictions based on *in situ* hybridization studies that have shown that SGK1 is not expressed in astrocytes (Wärntges *et al.* 2002). Instead, GluR6 abundance was enhanced by DEX at neurones in hippocampal CA3 and

dentate gyrus neurones, but not in the CA1 region. For a more detailed illustration of GluR6 expression on hippocampal neurones, we also stained primary hippocampal neurones with GluR6 antibody. As shown in Fig. 2, neuronal GluR6 was expressed in the cell body and at punctate sites along the neuronal processes, most probably reflecting synaptic sites. Using the MAP-2 antibody, we could easily distinguish between neurones and astrocytes in these cultures. In hippocampal neurones treated with DEX and TGF-β, the processes often showed an increase of GluR6 protein expression (Fig. 2, arrowheads). Again, no increase was observed in astrocytes.

In view of its effect on GluR6 expression, DEX may similarly increase the postsynaptic current of primary hippocampal neurones. To address this question whole-cell patch-clamp experiments from hippocampal neurones were performed. To avoid upregulation of SGK by serum, experiments were performed in hippocampal neurones exposed to serum-deprived culture medium 19–24 h prior to the electrophysiological experiments.

Steady-state inward currents were evoked in whole-cell voltage-clamped hippocampal neurones (holding potential (V_h) = −70 mV) by the agonist KA (100 μM). Prior to application of the agonist, the cells were incubated in 100 μM ConA to prevent desensitization of receptors. The KA-evoked current density was 10.1 ± 1.8 pA pF^{-1} ($n = 6$). It is known that KA can activate not only KARs but also AMPA receptor subunits (Hampson & Manalo, 1998). Thus, additional experiments were performed in the presence of NBQX, a known blocker of AMPA receptors. NBQX (1 μM) specifically blocks AMPA receptor subunits, whereas at higher concentrations (100 μM) NBQX blocks both AMPA- and KA-activated current components (Bureau *et al.* 1999). To verify that only AMPA receptor current was blocked by NBQX, we also used the AMPA receptor antagonist SYM 2206, which has been shown to selectively block AMPA receptors but not KARs at a concentration of 100 μM (Li *et al.* 1999; Bleakman *et al.* 2002). As shown in Fig. 3, 1 μM NBQX and 100 μM SYM 2206 were similarly effective. Mean current

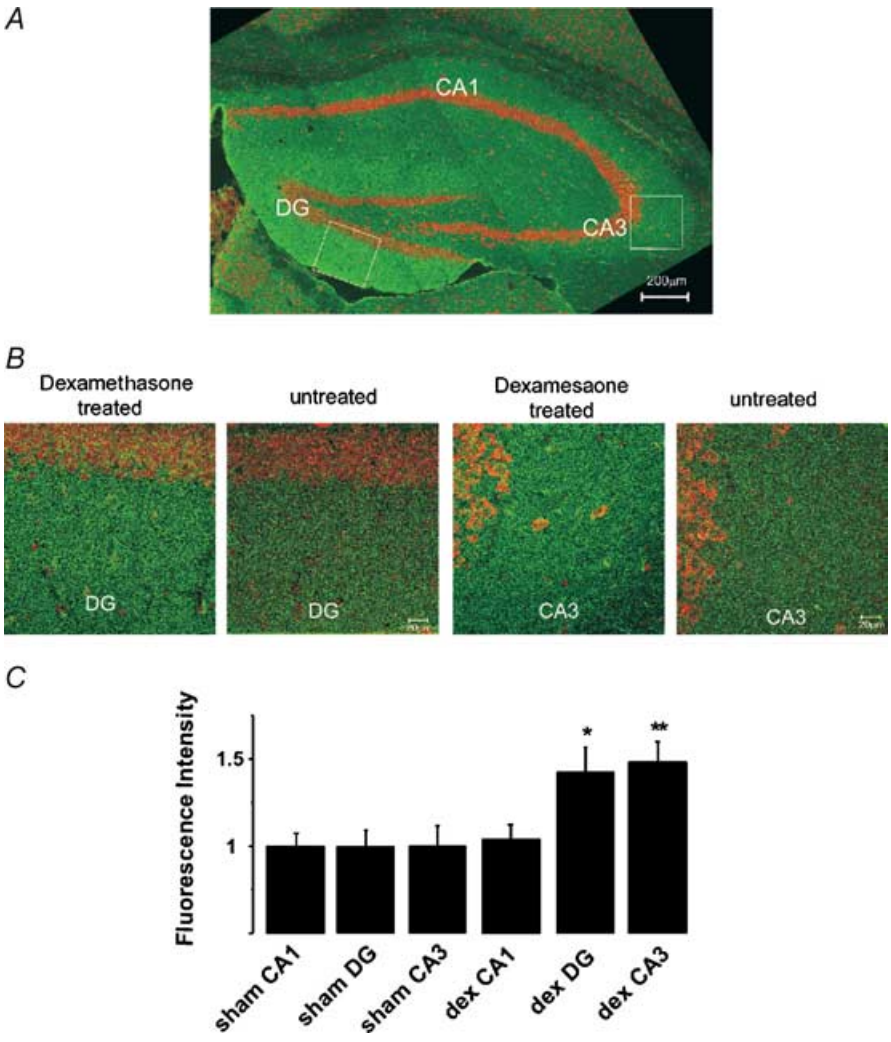

Figure 1. Anti-GluR6 antibody staining (green) of sections from the hippocampal region in control (untreated/sham) and dexamethasone (DEX)-treated mice
Fluorescent Nissl counterstain is shown in red. *A*, overview in a composite scan showing that GluR6 is expressed in all regions of the hippocampus, predominantly in the CA3 region and the dentate gyrus. This is true for both sham (untreated) and DEX-treated mice. The white rectangles indicate the approximate regions (CA3 and dentate gyrus, DG) of images shown in *B*. *B*, representative image pairs of sibling-matched sham (untreated) and DEX-treated mice from the DG (left) and the CA3 region (right) reveal that staining intensity is higher in the DEX-treated sibling. Such an increase was not seen in the CA1 region. *C*, arithmetic means of pixel intensities measured in a 10 × 10 × 3.5 μm volume in image stacks, as shown in *B*, in sections stained from three independent antibody stains from four pairs of sibling-matched mice. Data were normalized to the matched sham (untreated) image from the same stain and sibling and then pooled. Significant change is indicated by $^{**}P < 0.01$ and $^{*}P < 0.05$.

density was 0.6 ± 0.1 ($n = 6$) and 0.8 ± 0.2 pA pF^{-1} ($n = 6$) after KA + NBQX and after KA + SYM 2206 application, respectively. The following recordings were made in the presence of 1 μM NBQX.

To test for the possible involvement of DEX and TGF-β in the regulation of KAR currents, DEX or the combination of DEX and TGF-β was applied to the medium which was depleted of serum 19–24 h prior to recording. As shown in Fig. 3, incubation of hippocampal neurones with DEX (1 μM) alone, or DEX (1 μM) plus TGF-β (1 μM), in serum-free medium increased KAR-mediated current amplitudes significantly compared with untreated cells. The current density was enhanced fourfold after stimulation of cells with DEX (2.6 ± 0.6 pA pF^{-1}, $n = 10$) and 5.2-fold after stimulation with DEX + TGF-β (3.4 ± 1.1 pA pF^{-1}, $n = 13$) (Fig. 3), an effect paralleling the increase of GluR6 protein abundance on the cell surface (Fig. 2).

These observations provide clear evidence that GluR6 is regulated by glucocorticoid hormones. Additional experiments were performed to explore the possible involvement of the serum- and glucocorticoid-inducible kinase. SGK1 has previously been shown to be expressed in hippocampus by *in situ* hybridization in rats (Tsai *et al.* 2002). To test whether the same is true for mice, we performed RT-PCR of mouse hippocampal tissue. As shown in Fig. 4, SGK1 RNA is indeed expressed in the hippocampus of mice.

To test for a functional link between SGK1 and GluR6, further experiments were performed using the *Xenopus* oocyte expression system. To this end, the rat KAR subunit GluR6 has been expressed in *Xenopus* oocytes with or without coexpression of SGK1, SGK2 or SGK3. As illustrated in Fig. 5, the protein abundance of GluR6 is significantly enhanced in *Xenopus* oocytes expressing GluR6 together with SGK1, as compared with the GluR6

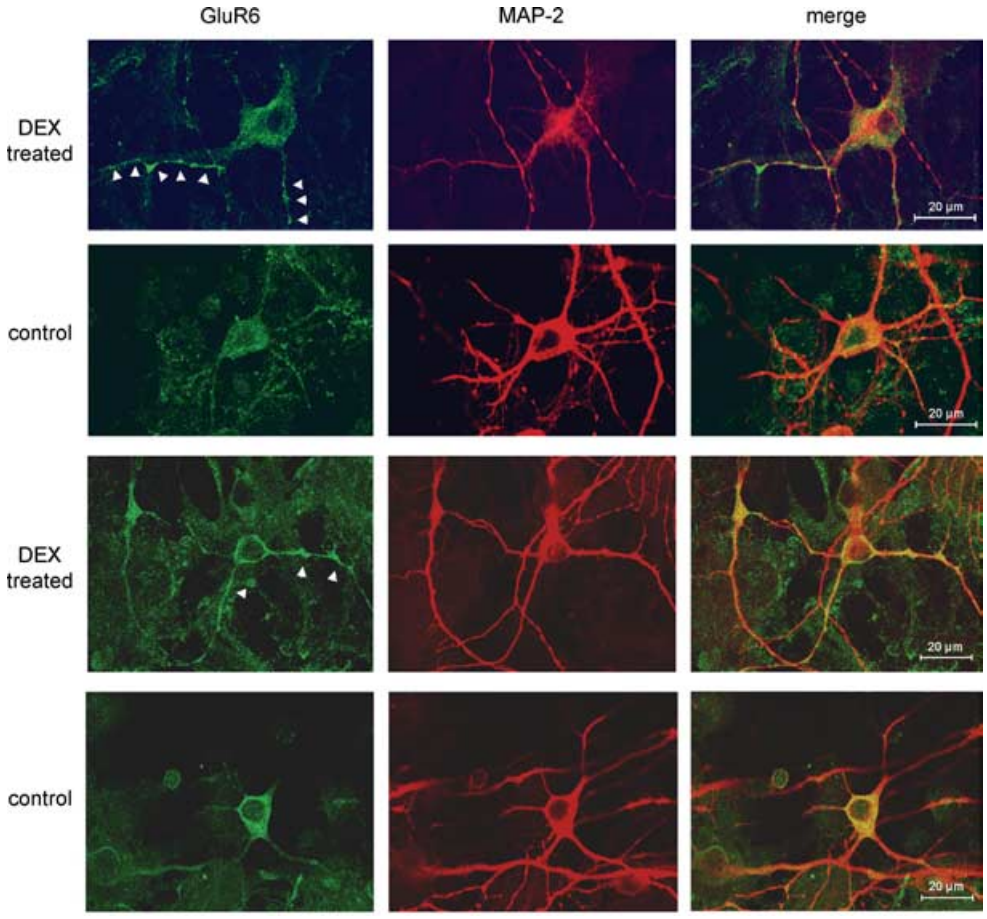

Figure 2. Anti-GluR6 (green) and microtubule-associated protein-2 (MAP-2) (red) antibody staining of primary hippocampal neurones
Two examples of neurones from DEX/tumour growth factor β (TGF-β)-treated and untreated cultures are shown in these confocal images generated from image stacks (total stack thickness approximately 5 μm). Neuronal processes are indicated by MAP-2 antigenicity. In cultures treated with DEX/TGF-β, many neuronal processes show clusters of GluR6 antibody reaction (white arrowheads). Those clusters are encountered less frequently in untreated control cultures. Scale bar, 20 μm.

protein abundance in oocytes expressing GluR6 alone. A smaller but still statistically significant effect on GluR6 protein abundance was observed following coexpression of SGK2 or SGK3, while coexpression with the related PKB was without significant effect. As shown in a different study by our group, SGK1 has no effect on the AMPA receptor subunit GluR1 (Strutz-Seebohm *et al.* 2005).

Using the two-electrode voltage-clamp technique, we compared glutamate-induced current amplitudes of oocytes injected with GluR6 cRNA alone compared with oocytes injected with GluR6 and SGK1, 2 or 3, or PKB. Similar to protein abundance, glutamate-induced current was significantly larger in *Xenopus* oocytes expressing GluR6 together with SGK1 than in *Xenopus* oocytes expressing GluR6 alone (Fig. 6). Again, SGK2 and SGK3 similarly stimulated the current, but were significantly less effective than SGK1. The PKB used in all experiments was always a constitutively active mutant (T308D/S473D).

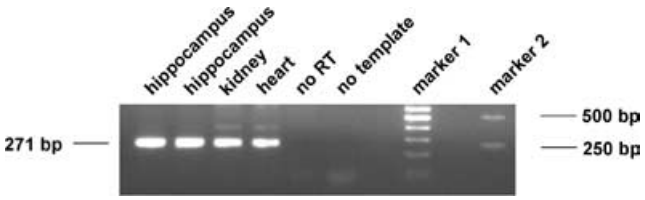

Figure 4. Expression of *Sgk1* in mouse hippocampal tissue
Hippocampal *Sgk1* mRNA was transcribed to cDNA and then quantified by RT-PCR. RNA from kidney and heart served as positive controls. Samples without reverse transcriptase (RT) and without template served as negative controls.

To assure that also the SGK kinases are active in the oocyte system, we repeated the experiments with the constitutively active SGK1(S422D) and SGK3(S419D), as well as inactive SGK1(K127N) and SGK3(K124N) mutants. The results showed that wild-type SGK1 and wild-type SGK3 are active when injected into oocytes.

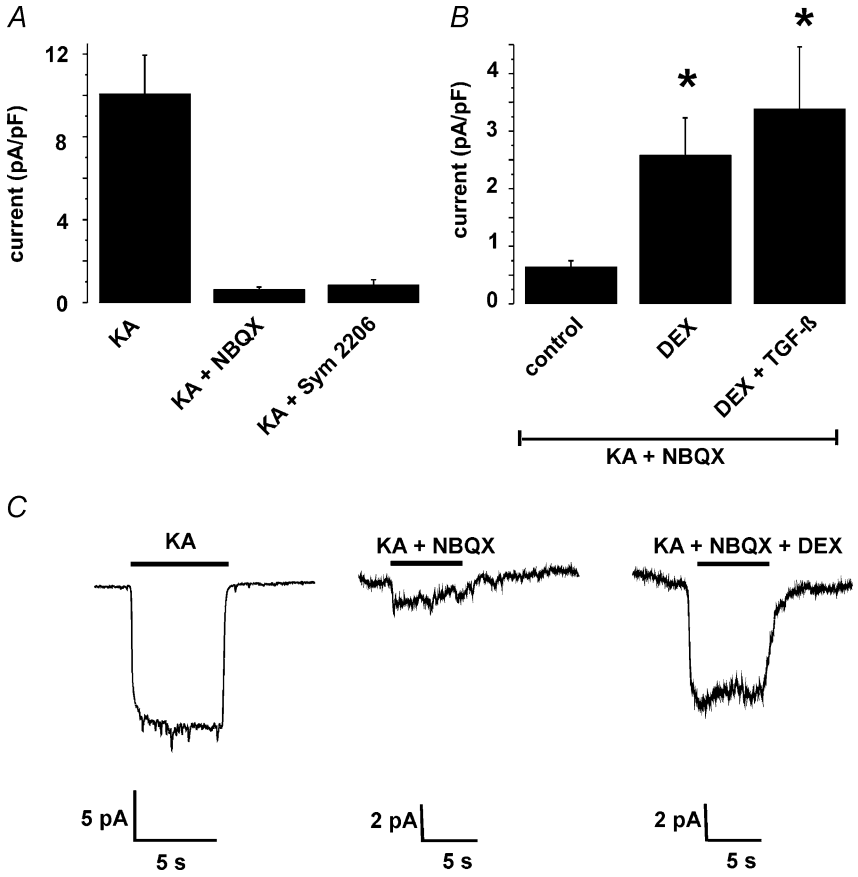

Figure 3. Upregulation of GluR6 current amplitudes by DEX in primary hippocampal neurones
A, responses of hippocampal neurones to kainate (KA; 100 μM; $n = 6$), KA (100 μM) + 6-nitro-7-sulfamoylbenzo (*f*)quinoxaline-2,3-dione (NBQX) (1 μM) ($n = 6$), KA (100 μM) + SYM 2206 (100 μM) ($n = 6$). Bar graphs show mean current densities at −70 mV. *B*, mean density of KA (100 μM) + NBQX (1 μM)-induced current in control conditions (serum deprivation, 20 h, $n = 6$) and after stimulation with DEX (1 μM, 20 h, $n = 10$) or with DEX (1 μM, 20 h) + TGF-β (1 μM, 20 h) ($n = 13$). *Significant ($P < 0.05$) difference from control. *C*, representative patch-clamp recordings of hippocampal neurone responses to KA (100 μM) and KA (100 μM) + NBQX (1 μM) in control conditions (serum deprivation, 20 h, left) and after stimulation with DEX (20 h, 1 μM, right).

Coexpression of SGK1(S422D) with GluR6 led to a current amplitude that was comparable with that of wild-type SGK1 (2.5 ± 0.2 and 3.0 ± 0.4 nA, respectively; $n = 9$–10), whereas coexpression of GluR6 with the inactive form SGK1(K127N) resulted in reduced current amplitudes (0.4 ± 0.2 nA, $n = 10$). For SGK3, no effect was seen for either the active (1.0 ± 0.2 nA, $n = 10$) or the inactive form (0.6 ± 0.2 nA, $n = 9$) compared with wild-type SGK3 (0.9 ± 0.2 nA, $n = 10$).

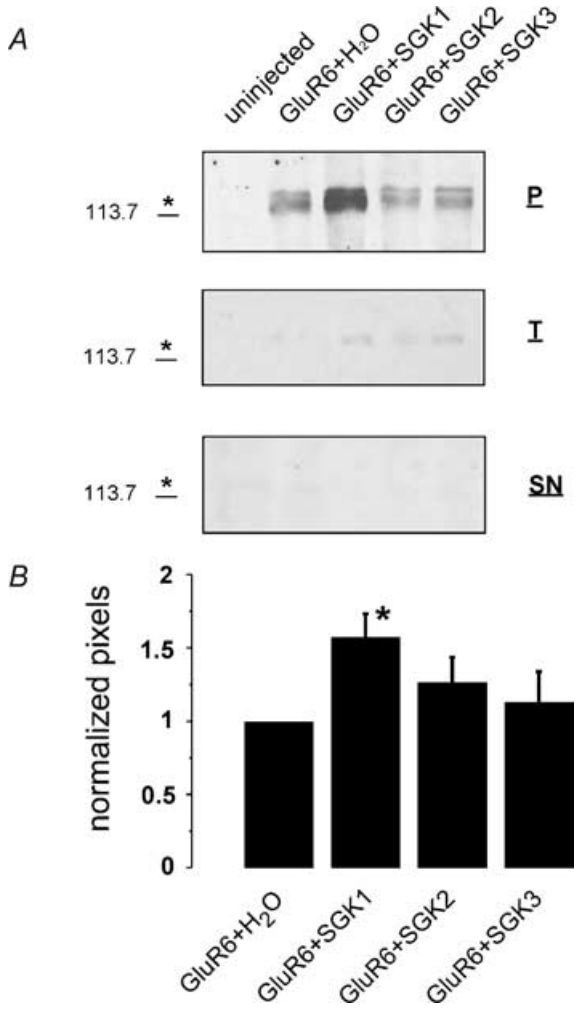

Figure 5. Western blot demonstrating SGK-regulated surface protein expression of the GluR6 subunit
A, glycosylated plasma membrane proteins expressed in oocytes were labelled with biotinylated concanavalin A (ConA). Oocytes were homogenized and plasma membrane proteins were streptavidin-precipitated. Samples including controls from uninjected oocytes were separated on a SDS gel, Western-blotted, and probed with an immunoaffinity purified antibody directed against a 16-amino-acid fragment of a C-terminus of GluR6. GluR6 protein has an apparent molecular mass of ~119 kDa (*). P, plasma membrane protein ($n = 18$); SN, supernatant fraction containing intracellular protein ($n = 1$); T, total protein ($n = 1$). *B*, bar graph showing relative abundance of GluR6 plasma membrane protein ($n = 18$). The band intensity was quantified by arithmetic analysis using the software Scion Image. The values of three different blots from different batches were used for statistical analysis. *Significant ($P = 0.05$) difference to staining intensity after expression of GluR6 alone. Due to the saturating conditions of the Western Blot, the analysis represents only an estimation of relative abundance of GluR1 plasma membrane protein.

Discussion

The present observations reveal a novel mechanism in the regulation of the GluR6 subunit of KARs. As apparent from functional studies in GluR6-deficient mice (Mulle *et al.* 1998; Bureau *et al.* 1999), KARs assembled with the GluR6 subunit are important for the sensitivity of CA3 and CA1 pyramidal neurones to KA and domoate (Bureau *et al.* 1999). In that study, the potential use of NBQX as a selective AMPA receptor antagonist was tested. The results implied that 1 μM NBQX is a fairly selective AMPA receptor antagonist that can be used to unmask KAR responses. In our study, we used NBQX, and also the antagonist SYM 2206, which has been

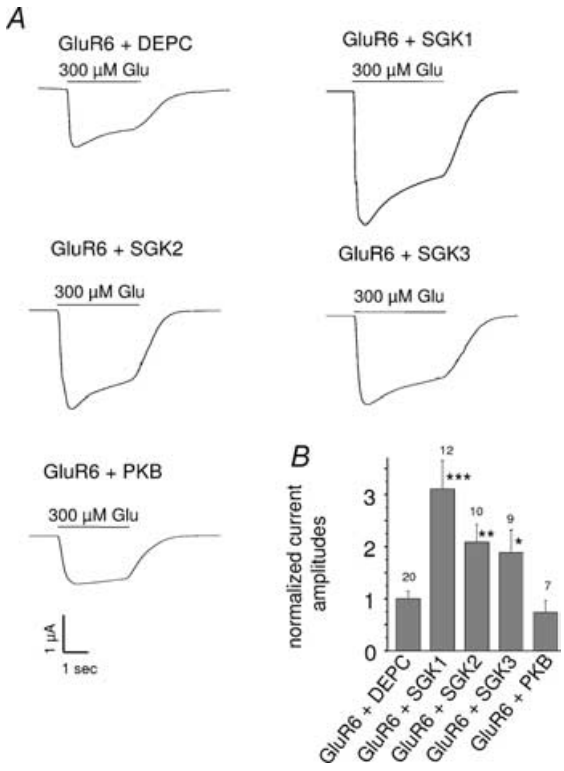

Figure 6. Increase in GluR6 currents by SGK isoforms
A, representative current traces measured in *Xenopus* oocytes in response to superfusion with 300 μM glutamate. All currents were measured at −70 mV and after pretreatment of oocytes with ConA to minimize desensitization. *B*, GluR6 current amplitudes in oocytes expressing GluR6 + diethylpyrocarbonate (DEPC)-H_2O ($n = 20$), GluR6 + SGK1 ($n = 12$), GluR6 + SGK2 ($n = 10$), GluR6 + SGK3 ($n = 9$) and GluR6 + protein kinase B (PKB) ($n = 7$) were measured and are shown normalized to the GluR6 + DEPC-H_2O currents. Significant differences from current amplitudes in oocytes expressing GluR6 alone are indicated by ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

shown to selectively block AMPA but not KARs at a concentration of 100 μM (Li *et al.* 1999; Bleakman *et al.* 2002), and found no significant differences in amplitudes after coapplication of KA and one of the antagonists. The experiments were performed after pretreatment of cultures with ConA to remove receptor desensitization (Huettner, 1990; Wong & Mayer, 1993; Bleakman *et al.* 2002). Since GluR5-mediated inward currents are absent in young hippocampal neuronal cultures (Bleakman *et al.* 1999) and as GluR7 receptors require high concentrations of KA (>1 mM) for activation (Schiffer *et al.* 1997) and play a negative modulatory role in heteromeric KARs (Strutz *et al.* 2001), it appears safe to conclude that the currents observed at the agonist concentrations of 100 μM are carried by the GluR6 receptor. Although the high-affinity KARs KA1 and KA2 are also expressed in hippocampal neurones, there has been no indication so far that they form functional homomeric or heteromeric (KA1–KA2) channels. However, GluR6 forms homomeric or heteromeric channels with GluR5, GluR7 (Dingledine *et al.* 1999), or KA1 or KA2 (Schiffer *et al.* 1997), and the measured currents may be due to homomerically expressed GluR6 and/or heteromeric GluR6 complexes. Nevertheless, our immunohistochemistry data, as well as our patch-clamp data, indicate that GluR6 membrane protein is increased after administration of DEX or DEX and TGF-β. Whether or not the receptor is homomerically or heteromerically expressed remains unclear.

A good candidate to mediate the effect of DEX on GluR6 is the serum- and glucocorticoid-inducible kinase. The *Sgk* gene was originally cloned as a glucocorticoid-inducible gene from rat mammary tumour cells (Webster *et al.* 1993*b*), and since then it has been demonstrated to be regulated by glucocorticoids in a variety of cells and tissues (Brennan & Fuller, 2000; Naray-Fejes-Toth *et al.* 2000; Helms *et al.* 2003; Wu *et al.* 2004). In contrast to SGK1, the isoforms SGK2 and SGK3 are not genomically regulated by glucocorticoids (Lang & Cohen, 2001). All three SGK isoforms are activated by a signalling cascade involving PI3 kinase and phosphoinositide-dependent kinase PDK1 (Lang & Cohen, 2001). The cascade is triggered by oxidative stress, insulin and growth factors including NGF, BDNF, TGF-β and IGF1 (Lang & Cohen, 2001). Interestingly, DEX alone proved to be similarly effective on hippocampal neurones than DEX and TGF-β added together. Apparently, the activation of SGK1 is not limiting. Accordingly, similar to earlier observations (Lang *et al.* 2003; Pearce, 2003; Verrey *et al.* 2003), coexpressed SGK1 was effective in *Xenopus* oocytes even in the absence of exogeneous activators of PI3 kinase.

SGK1 differs from its isoforms by its exquisite sensitivity to genomic regulation. It appears that *Sgk1* is not required for basic functions, but mediates the upregulation of function during stress conditions. *sgk* is also known as a primary glucocorticoid-induced gene in several cell lines studied in humans (Naray-Fejes-Toth *et al.* 2000). Relatively little is known about the functional role of SGK1 in the central nervous system. In an earlier study, *sgk1* mRNA levels were found to be increased at the lesion site after brain injury, suggesting that *sgk1* may be involved in axonal regeneration (Imaizumi *et al.* 1994). Most interestingly, the kinase has been implicated in the generation of memory, since, in rats, SGK1 expression correlated with the ability to learn (Tsai *et al.* 2002). The strong effect of SGK1 on GluR6 protein abundance in the cell membrane could indeed contribute to neuronal excitability and memory consolidation.

In conclusion, we demonstrate that the glucocorticoid DEX upregulates GluR6 protein abundance in both cerebral tissue and isolated hippocampal neurones. We further demonstrate that DEX enhances KA-induced currents in hippocampal neurones. Finally, we provide evidence for a strong effect of the DEX-regulated kinase SGK1 on GluR6 protein abundance in the cell membrane and GluR6-mediated KA-induced currents in oocytes. Although abundance and activation of the kinases, and differences of efficacy in oocytes, may be different from those in neuronal cells, we propose that the enhancement of cerebral KAR density and function by glucocorticoids is an effect at least partially due to SGK1.

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